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Nutrient regulation of transcription and signalling by O-GlcNAcylation☆

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Summary The cycling (addition and removal) of O-linked N-acetylglucosamine (O-GlcNAc) on serine or threonine residues of nuclear and cytoplasmic proteins serves as a nutrient sensor via the hexosamine biosynthetic pathway's production of UDP-GlcNAc, the donor for the O-GlcNAc transferase (OGT). OGT is exquisitely sensitive both in terms of its catalytic activity and by its specificity to the levels of this nucleotide sugar. UDP-GlcNAc is a major node of metabolism whose levels are coupled to flux through the major metabolic pathways of the cell. O-GlcNAcylation has extensive crosstalk with protein phosphorylation to regulate signalling pathways in response to flux through glucose, amino acid, fatty acid, energy and nucleotide metabolism. Not only does O-GlcNAcylation compete for phosphorylation sites on proteins, but also over one-half of all kinases appear to be O-GlcNAcylated, and many are regulated by O-GlcNAcylation. O-GlcNAcylation is also fundamentally important to nutrient regulation of gene expression. OGT is a polycomb gene. Nearly all RNA polymerase II transcription factors are O-GlcNAcylated, and the sugar regulates their activities in many different ways, depending upon the transcription factor and even upon the specific O-GlcNAc site on the protein. O-GlcNAc is part of the histone code, and the sugar affects the modification of histones by other epigenetic marks. O-GlcNAcylation regulates DNA methylation by the TET family of proteins. O-GlcNAc modification of the basal transcription machinery is required for assembly of the pre-initiation complex in the transcription cycle. Dysregulated O-GlcNAcylation is directly involved in the aetiology of the major chronic diseases associated with ageing.

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Contents

Introduction

The modification of nuclear and cytoplasmic proteins by O-linked N-acetylglucosamine (O-GlcNAc) was discovered about thirty years ago (Torres and Hart, 1984; Holt and Hart, 1986). Until then, it was widely regarded not only in textbooks, but also by glycobiology experts, that protein glycosylation only occurred in the lumenal or extracellular compartments of cells. Early studies established that O-GlcNAcylation is particularly abundant within the nucleus (Holt and Hart, 1986; Holt et al., 1987a; Park et al., 1987; Kelly and Hart, 1989), but also occurs on many cytoskeletal and other cytosolic proteins (Holt et al., 1987b; Hart et al., 1988). Studies also showed that O-GlcNAc cycles rapidly on proteins in response to stimuli, suggesting that it is a regulatory modification analogous to protein phosphorylation (Kearse and Hart, 1991; Roquemore et al., 1992). Work from several laboratories in the past three decades have shown that not only is O-GlcNAc amongst the most abundant and wide-spread of post-translational modifications, but also that the cycling sugar serves as a nutrient sensor to regulate nearly all aspects of cellular physiology (for recent reviews: Hart et al., 2011; Slawson and Hart, 2011; Hardiville and Hart, 2014) (Fig. 1). OGT is essential in mammals and plants, even at the single cell level (Shafi et al., 2000; Hartweck et al., 2002; O'Donnell et al., 2004; Olszewski et al., 2010). Highlights of its many functions, which depend upon protein and even upon the sites on the protein to which the sugar is attached, include: (1) It is essential for both B- and T-lymphocyte activation (Golks et al., 2007). (2) It regulates many protein—protein interactions (Lim and Chang, 2009a,b; Roos et al., 1997; Hiromura et al., 2003; Wells et al., 2011). (3) Nutrients regulate our circadian clocks via the cycling of O-GlcNAc on transcription factors (Durgan et al., 2011; Kim et al., 2012; Hart, 2013; Kaasik et al., 2013). (4) Multiple subunits of the proteasome are O-GlcNAcylated and the sugar regulates the activity of this degradation complex (Zhang et al., 2003; Liu et al., 2004; Bowe et al., 2006). (5) O-GlcNAc has a dynamic interplay with phosphorylation, ubiquitination and other key regulatory protein modifications, allowing nutrients to regulate known signalling pathways (Wang et al., 2010a; Zeidan and Hart, 2010; Trinidad et al., 2012; Ruan et al., 2013). (6) O-GlcNAcylation plays a direct role in neuronal functions, including learning and memory and synaptic vesicle trafficking (Trinidad et al., 2012; Cole and Hart, 1999, 2001; Lagerlof and Hart, 2014; Vosseller et al., 2006; Francisco et al., 2009; Tallent et al., 2009; Skorobogatko et al., 2014; Trinidad et al., 2013). (7) O-GlcNAc cycling regulates growth hormone signalling in plants (Scott et al., 2006). (8) Increased O-GlcNAcylation protects cells from acute stresses, such as heat, high salt, ultraviolet light and hypoxia, among others (Zachara et al., 2004; Slawson et al., 2006; Cheung and Hart, 2008; Zachara, 2012). (9) O-GlcNAcylation also regulates translation and ribosome biogenesis (Ohn et al., 2008; Zeidan et al., 2010), but much more work in this area is needed. (10) Nutrients regulate transition through the cell cycle by O-GlcNAcylation and abnormal increases in OGT lead to polyploidy, a common feature of cancer cells (Wang et al., 2010a,b; Slawson et al., 2005, 2008). (11) O-GlcNAcylation is up-regulated in all cancers examined to date, and in chronic lymphocytic leukaemia the extent of O-GlcNAcylation of the leukocytes correlates with patient prognosis (Slawson and Hart, 2011; Chou and Hart, 2001; Caldwell et al., 2010; Slawson et al., 2010; Yi et al., 2012; Ma and Vosseller, 2013; Shi et al., 2010; Lynch et al., 2012; Ma and Vosseller, 2014). (12) Decreased O-GlcNAcylation, due to reduced glucose utilization in the brain, is directly involved in the aetiology of Alzheimer's disease, and inhibitors of O-GlcNAcase show promise as therapeutics (Yuzwa and Vocadlo, 2014; Zhu et al., 2014; Dias and Hart, 2007; Arnold et al., 1996). This list represents only a few of the many functions of this ubiquitous protein modification, which thus far has been reported on over four thousand proteins. However, the number of nuclear and cytoplasmic proteins modified is likely much larger. In this short review, I will focus on the fundamental importance of O-GlcNAcylation in transcription and also on its ubiquitous importance to nutrient modulation of cellular signalling.

O-GlcNAcylation is fundamentally important to nutrient regulation of transcription

Some of the earliest work on O-GlcNAcylation showed that it is highly enriched in chromatin and visualization of *Drosophila* polytene chromosomes showed that the sugar is localized at active sites of transcription (Kelly and Hart, 1989). The IIa form of RNA polymerase II, which is the form of the enzyme involved in initiation of transcription, was found to be extensively O-GlcNAcylated on its C-terminal (CTD) domain (Kelly et al., 1993). O-GlcNAcylation of the CTD is mutually exclusive to phosphorylation of the CTD, which produces the RNA polymerase IIo isoform, the form of the enzyme involved in elongation (Comer and Hart, 2000, 2001). Using more modern methods, like high-throughput ChIP analyses in both mammalian cells and *Caenorhabditis elegans*, both cycling enzymes, OGT and OGA, as well as O-GlcNAc itself, are found to be highly localized at the start sites of thousands of genes (Lewis and Hanover, 2014; Ranuncolo et al., 2012). In a B lymphocyte cell line, partial knock down of OGT by shRNA, strikingly reduces the

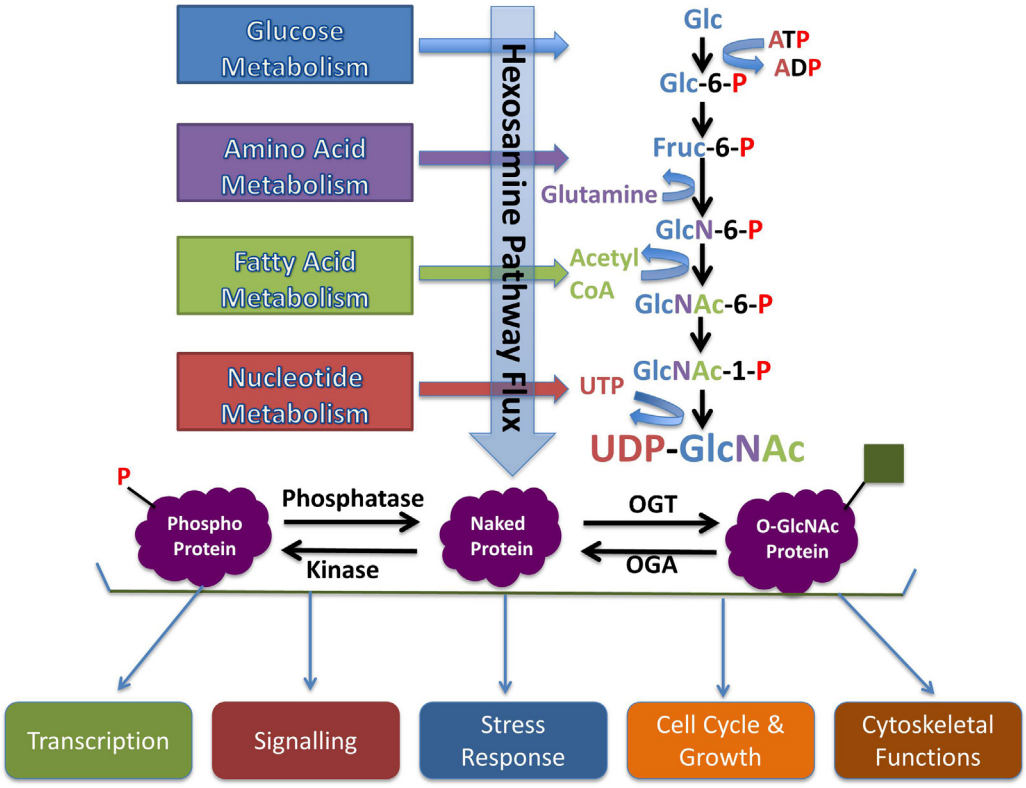

Figure 1 O-GlcNAcylation serves as a nutrient sensor to regulate many processes of cell physiology. UDP-GlcNAc, the high-energy sugar donor for O-GlcNAcylation is a major node of metabolism. In fact, flux through glucose-, amino acid-, fatty acid- and nucleotide-metabolism all directly affect the concentrations of UDP-GlcNAc formed by the hexosamine biosynthetic pathway. In turn, the O-GlcNAc transferase (OGT) is exquisitely sensitive the concentrations of UDP-GlcNAc in terms of its activity and its specificity. Removal of O-GlcNAc is catalyzed by the enzyme O-GlcNAcase (OGA). The interplay of O-GlcNAc cycling and phosphate cycling on myriad cytoplasmic and nuclear proteins regulates nearly every aspect of cellular physiology.
Modified after Hart et al. (2011).

transcription of many genes, concomitant with reduced binding of the transcription machinery to the promoters. In an in vitro transcription system using the adenoviral E3 promoter and HeLa cell nuclear extracts, inhibitors of OGT and OGA blocked transcription, but only if they are added prior to initiation, but not after elongation has begun (Lewis and Hanover, 2014; Ranuncolo et al., 2012; Lewis, 2013). These data strongly suggest that O-GlcNAcylation is required for assembly of the transcription complex at the promoter, and its removal is required for elongation to take place, a model proposed nearly twenty-years ago (Comer and Hart, 1999). The recent discovery that OGT is a polycomb gene, which regulates large sets of developmentally important genes, such as HOX genes, further supports the fundamental importance of O-GlcNAc cycling in transcriptional regulation (Myers et al., 2011; Sinclair et al., 2009; Gambetta et al., 2009).

Nearly all RNA polymerase II transcription factors are extensively O-GlcNAcylated (Ozcan et al., 2010). Among the earliest shown to be O-GlcNAcylated is the transcription factor, Sp1, which is not only one of the most extensively modified of all O-GlcNAcylated proteins, but also is one of the most important 'housekeeping' transcription factors in cells (Jackson and Tjian, 1988). O-GlcNAcylation of Sp1 may be important to glucose toxicity and abnormal gene expression in certain tissues in diabetes (Donovan et al., 2014; Majumdar et al., 2004). The presence of O-GlcNAc on transcription factors was often used to purify them by lectin-affinity chromatography (Jackson and Tjian, 1989). O-GlcNAc regulates the functions of transcription factors in almost every conceivable way, depending upon the site and transcription factor, such as by regulating nuclear localization, DNA binding (Kang et al., 2013), turnover (Kaasik et al., 2013; Ruan et al., 2013), transactivation (Ramakrishnan et al., 2013) and interactions with other components of the transcription machinery (Lim and Chang, 2009a,b,c; Hiromura et al., 2003). Host cell factor 1 (HCF-1), which has more than twelve O-GlcNAc sites, regulates mitosis, the cell cycle and is key to Herpes viral infections (Myers et al., 2013). Remarkably, OGT not only extensively modifies HCF-1, but also by using UDP-GlcNAc at is active site as part of the molecular mechanism, it acts as a protease to cleave the protein into multiple fragments, each with unique functions (Lazarus et al., 2013). It is possible that this unique proteolysis mechanism might also occur on other glycosyltransferases? Nutrients help to fine tune our circadian clocks by using the interplay between O-GlcNAcylation, phosphorylation and ubiquitination to control the transcription cycles that comprise the clock (Durgan et al., 2011; Kim et al., 2012; Hart, 2013; Kaasik et al.,

2013). NFκB, the most important transcription factor regulating cells of our immune system, is regulated via multiple mechanisms that involve O-GlcNAcylation of its specific subunits (Ramakrishnan et al., 2013; Allison et al., 2012). O-GlcNAcylation is part of the mechanism that allows different receptors to share the same signalling pathway, but still result in activation of different genetic programmes. For example, in the same cells, signalling of the T-cell receptor (TCR) through NFκB requires O-GlcNAcylation of the c-Rel subunit, while signalling through NfkB by the tumour necrosis factor receptor (TNFR) does not require O-GlcNAcylation of the c-Rel subunit (Ramakrishnan et al., 2013). Nearly all of the components of the basal transcription machinery are O-GlcNAcylated and emerging evidence suggests that cycling of the sugar on the TATA-binding protein is key to nutrient regulation of TBP's interaction with DNA (Comer and Hart, 1999).

O-GlcNAc is part of the histone code with the sugar modification localized in the tail region of histones (Sakabe et al., 2010). However, some of the O-GlcNAc sites on histones are located at regions of the nucleosome in intimate contact with the DNA. O-GlcNAc cycles on histones with the cell cycle and with altered gene expression due to exposure to stress (Sakabe et al., 2010). Histone O-GlcNAcylation also appears to regulate other histone marks (Sakabe and Hart, 2010). Strikingly, enzymes that methylate DNA to form 5-hydroxymethylcytosine, an important epigenetic mark, not only target OGT to chromatin, but also are themselves regulated by O-GlcNAcylation (Chen et al., 2013; Deplus et al., 2013; Zhang et al., 2014). It is now clear that O-GlcNAc is not only a bona fide epigenetic modification on its own, but also that modest increases in OGT by a twofold overexpression in cells, dramatically alters the modification of histones by other well-studied epigenetic modifications at key sites on histones, including phosphorylation, methylation and acetylation (Sakabe and Hart, 2010). Thus, while we know very little about the mechanistic functions of specific O-GlcNAcylation on proteins involved in transcription, it is now abundantly clear that research in this area is critical to nearly every aspect of our understanding of the molecular processes controlling gene expression.

Nutrient regulation of signalling occurs by extensive crosstalk between O-GlcNAcylation and phosphorylation

At present, thousands of O-GlcNAcylation sites have been mapped on proteins (Hart et al., 2007, 2011). Virtually all of these sites are on so-called unstructured loops when the crystal structure is known. Since crystallographers remove all post-translational modifications when crystallizing proteins, it seems likely that these regulatory loops do indeed have specific structures when they are actually modified as they are in cells. On many proteins, the same hydroxyl group on the polypeptide is reciprocally occupied by either a phosphate or O-GlcNAc, depending upon the cellular status (Wang et al., 2010a; Zeidan and Hart, 2010; Trinidad et al., 2012; Hart et al., 1996; Copeland et al., 2008). For example, the wild-type c-Myc oncoprotein, alternatively either has a phosphate or a O-GlcNAc at Thr58, which is the site most mutated in human lymphomas that cause this transcription factor to become an oncoprotein. In non-growing cells Thr58 of c-Myc is O-GlcNAcylated, but rapidly becomes phosphorylated when cells are stimulated to divide (Chou et al., 1995a,b; Kamemura et al., 2002). There are many proteins where the O-GlcNAc and phosphate are not at the exact same site, yet the population of molecules that are phosphorylated do not contain O-GlcNAc and vice versa (Chou et al., 1992). There are other proteins, such as insulin receptor substrates (IRS), which contain many sites of both phosphorylation and O-GlcNAcylation simultaneously at different sites and reciprocally at shared sites (Park et al., 2005; Ball et al., 2006; Klein et al., 2009). Given the critical importance of IRS proteins in insulin and growth factor signalling, it is not surprising that these proteins are regulated in such a complex way both nutrients and energy-dependent phosphorylation. Given the abundance of both phosphorylation and O-GlcNAcylation and the fact that they compete for the same residues on proteins, it is not surprising that the cell would use their interplay to tune signalling processes in response to nutrients.

Proteomic analyses revealed how extensive this crosstalk between phosphorylation and O-GlcNAcylation actually is. Treating cells with an O-GlcNAcase inhibitor elevates global O-GlcNAcylation about threefold. Quantitative analysis of phosphorylation occupancy at seven hundred sites showed that nearly every actively cycling site of phosphorylation was affected, either up or down, by altering the global O-GlcNAc levels (Wang et al., 2008). Decreased phosphorylation when O-GlcNAcylation was increased was expected from the presumed reciprocal relationship of the two modifications. Why then did phosphate site occupancy at one hundred and forty-eight sites increase when global O-GlcNAcylation was increased? Of course, we now know that many, if not most kinases are not only modified by O-GlcNAcylation, but also several studies have shown that kinases are indeed regulated by the sugar modification. OGT associates with protein phosphatases, indicating that the same enzyme complex may remove the phosphate and attach the sugar without releasing the polypeptide (Wells et al., 2004). Both OGT and OGA are regulated by phosphorylation (Song et al., 2008; Bullen et al., 2014; Alonso et al., 2014). Thus, the crosstalk between phosphorylation and O-GlcNAcylation is not only due to competition at the level of site occupancy on polypeptides, but also is controlled by each modification regulating the other's cycling enzymes.

Many kinases are dynamically O-GlcNAcylated, and recent analyses using protein arrays suggest that over one-half of all kinases are modified by the sugar (Dias et al., 2012). O-GlcNAcylation occurs on all families of kinases whether they are classified in terms of evolutionary relationship or functional types. Thus far, every O-GlcNAcylated kinase studied is regulated by the sugar in some fashion. For example, calcium calmodulin kinase IV (CAMKIV), which very important in regulation if neuronal functions and in pancreatic beta cell functions, is activated by calmodulin and phosphorylation at Thr200. Upon nerve cell depolarisation, phosphorylation of Thr200 on CAMKII increases, while O-GlcNAcylation at several sites, but most importantly at Ser189 decreases (Dias et al., 2009). Ser189 is located in the ATP-binding pocket of CAMKIV and occupancy by O-GlcNAc blocks binding of ATP to the kinase. O-GlcNAcase must remove the sugar before CAMKIV can be activated. In

turn, activated CAMKIV modifies OGT and activates it (Song et al., 2008), creating a cycle that regulates the activation of this important kinase. In contrast, CAMKII, which is a critical regulator of our cardiac contractility and is also important in nerve cells, is normally activated by phosphorylation of Thr286, which opens up the protein (Erickson et al., 2013). However, in diabetic hearts Ser279 becomes abnormally O-GlcNAcylated causing the enzyme to become constitutively active. This abnormal activation contributes directly to the arrhythmias associated with diabetic cardiomyopathy. In dividing and growing cells, OGT is mostly within the nucleus. However, in non-dividing differentiated cells, such as skeletal muscle and neurons, it is mostly cytoplasmic and its nuclear localization is regulated. The major energy sensing kinase of cells, adenosine monophosphate activated kinase (AMPK), phosphorylates OGT at Thr444 inducing its nuclear localization in skeletal muscle cells (Bullen et al., 2014). AMPK itself is O-GlcNAcylated on its alpha 1 and 2 and gamma regulatory subunits and global inhibition of O-GlcNAc cycling blunts the overall activation of AMPK. The casein kinase II (CKII) alpha subunit is O-GlcNAcylated at Ser347 proximal to its phosphorylation at Thr344 (Tarrant et al., 2012). Remarkably, using protein arrays to assay substrate specificity, the phosphorylated and O-GlcNAcylated isoforms were shown to have different substrate selectivity. This finding could have profound implications with respect to how individual modifications regulate signalling. Protein kinase C isoforms are regulated by O-GlcNAcylation (Matthews et al., 2005; Robles-Flores et al., 2008). Activation of AKT (protein kinase B) is also regulated by O-GlcNAcylation in certain cell types (Park et al., 2005; Gandy et al., 2006; Kang et al., 2008). O-GlcNAcylation of phosphofructokinase 1 (PFK-1), which is the key enzyme regulating glycolysis, regulates growth and metabolism, particularly of cancer cells, by diverting flux of glucose into the pentose phosphate pathway (Yi et al., 2012).

Conclusions and future directions

In the past three decades of research on O-GlcNAcylation, it has become clear that this ubiquitous sugar modification of proteins serves as a major nutrient sensor to regulate many aspects of cellular physiology (Fig. 1). The development of advanced enrichment and mass spectrometric methods to detect and quantify O-GlcNAc has played a major role in moving this field forward (Myers et al., 2013; Wang et al., 2010b; Alfaro et al., 2012). While methods and tools have improved (Zachara et al., 2011), there still is an acute need to develop better approaches to elucidate O-GlcNAc's functions. The availability of site-specific antibodies, like those available for phosphorylation, would greatly advance the field. Currently, the only methods to alter the levels of O-GlcNAc, either up or down, cause global changes. There is an acute need to be able to increase or decrease the modification of proteins by O-GlcNAc on individual proteins or even at individual sites to elucidate functions. As it is now clear that O-GlcNAcylation is important to the aetiology of cardiovascular functions and disease (Zachara, 2012; Marsh and Chatham, 2014; Karunakaran and Jeoung, 2010; Chatham and Marchase, 2010), cancer (Slawson and Hart, 2011; Caldwell et al., 2010; Slawson et al., 2010; Ma and Vosseller, 2013, 2014), diabetes (Hart et al., 2011; Vaidyanathan and Wells, 2014; Semba et al., 2014; Ma and Hart, 2013; Teo et al., 2010; Issad et al., 2010), and Alzheimer's disease (Yuzwa and Vocadlo, 2014; Zhu et al., 2014; Dias and Hart, 2007; Alfaro et al., 2012), thus, the need to have more researchers in this area are clearly justified. We will never understand the molecular mechanisms controlling signalling and transcription until the research community focuses much more of its efforts on O-GlcNAcylation, which in the past thirty years has largely been ignored by the mainstream, perhaps mostly due to the difficulties in detecting it and the lack of tools.

Conflict of interest

The author receives a share of royalty received by the university on sales of the CTD 110.6 antibody (pan-specific O-GlcNAc antibody), which are managed by JHU.

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